Existence of *Leptospira interrogans* in kidney and shedding from urine and relationship with histopathological and serological findings in water buffaloes (*Bubalus bubalis*)

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ABSTRACT

In order to detect *Leptospira interrogans* in both kidney and urine and its correlation with interstitial nephritis and serological results, samples of blood, urine and kidney were taken, immediately after slaughter, from 353 water buffaloes at Ahvaz abattoir in Khuzestan province, Iran. Sera were initially screened at serum dilution of 1:100 against seven live antigens of *Leptospira interrogans*: pomona, hardjo, ballum, icterohemorrhagiae, tarassovi, australis and grippotyphosa using the microscopic agglutination test (MAT). Sera with positive results were titrated against reacting antigens in serial twofold dilution from 1:100 to 1:800. The samples of kidney were embedded in paraffin wax and two 10µm thick sections were obtained for Polymerase chain reaction (PCR) examination. PCR was done on urine and kidney by using LipL32 gene primers. Antibodies against one or more serovars at dilution ≥:100 were detected in 43 (12.2%) of examined buffaloes; that is 26 (8.2%) of kidney tissues, 14 (4.8%) of urine samples separately and 3 (0.84%) of both kidney and urine samples were positive in PCR. From 153 (43.3%) buffaloes with positive MAT, 24 cases were positive by PCR of kidney and/or urine samples, synchronously. Renal lesions such as interstitial nephritis, acute tubular necrosis (ATN), pyelonephritis, glomerulonephritis, renal fibrosis and hydronephrosis were found in 128 (36.3%) cases. Statistical analysis indicated that there was no significant association between results of MAT with PCR.

Keywords: Leptospiral infection, PCR, MAT, water buffalo.

RÉSUMÉ

Présence de *Leptospira interrogans* dans rein et l’urine et relations avec les données histopathologiques et sérologiques chez le buffle d’eau (*Bubalus bubalis*)

Des prélèvements de sang, urine et reins ont été réalisés immédiatement après l’abattage de 353 buffles d’eau à l’abattoir d’Ahvaz dans la province de Khuzestan, Iran dans le but de corrélérer la présence de *Leptospira interrogans* dans les reins et l’urine et avec des lésions de néphrite interstitielle et les résultats sérologiques. Les sérums ont été initialement dépistés à une dilution de 1:100 contre sept antigènes vivants de *Leptospira interrogans* : Pomona, hardjo, ballum, icterohemorrhagiae, tarasovi, australis et grippotyphosa en utilisant le test d'agglutination microscopique (MAT). Les sérums avec des résultats positifs ont été titrés contre des antigènes réagissant avec aux dilutions de 1:100 à 1:800. Les échantillons de reins ont été inclus dans de la paraffine et coupés en tranches de 10 µm pour analyse par PCR. La PCR a été effectuée sur l’urine et les reins en utilisant les amorces de gène LipL32. Les anticorps dirigés contre un ou plusieurs sérovars à dilution ≥:100 ont été détectés dans les sérums. Le sérovor positif le plus fréquent était hardjo (56.2%), suivi par pomona (52.3%), australis (9.8%), tarassovi (5.9%), grippotyphosa (4.5%) et icterohemorrhagiae (3.9%). La présence de *L. interrogans* a été révélée par PCR chez 43 (12.2%) buffles, avec 26 (8.2%) tissus rénaux, 14 (4.8%) échantillons d’urine séparément positifs et 3 (0.84%) reins et urines simultanément positifs. Parmi les 153 des buffles ayant une réaction positive en MAT, 24 cas étaient positifs par PCR sur le rein et/ou l’urine. Des lésions rénales telles que la néphrite interstitielle, nécrose tubulaire aiguë (ATN), pyélonéphrite, glomérulonéphrite, fibrose rénale et hydronephrose ont été trouvées dans 128 (36.3%) cas. L’analyse statistique a indiqué qu’il n’y avait pas d’association significative entre les résultats de MAT et PCR.

Mots-clés : Infection Leptospirose, PCR, MAT, buffle d’eau

Introduction

Leptospirosis is a worldwide zoonotic disease and is most common in tropical and subtropical areas with high rainfall (18). The disease is caused by pathogenic leptospires which are classified into one species of *Leptospira interrogans* containing over 212 serovars and are arranged into 23 serogroups. pomona, hardjo, grippotyphosa and canicola are the commonest pathogenic serovars for all farm animals, but *L. interrogans* serovars hardjo and pomona are responsible for most bovine leptospirosis cases (16). The disease is common in cattle, buffalo, sheep, goat, dog and equine and causes fever, hemolysis, renal tubular necrosis (nephritis), reproductive disorders and death (9, 10). Bovine leptospirosis is dominantly subclinical, and excretion of the cause of disease through urine has a prominent role in disease distribution, amongst not only susceptible animal but also people at risk (5).

However, selection of suitable method for diagnosis of leptospiral infections is an arguable subject in veterinary medicine. Although, bacterial culture has a reliable result, the method is slow and laborious (7).

The microscopic agglutination test (MAT) is the most commonly used serological test for diagnosis of leptospirosis and provides information about the serovars in each areas (3, 16); it is however less useful in diagnosis the early state and chronic disease (16). Early diagnosis of leptospirosis is important because severe leptospiral infection can have a fulminant course. The available serological techniques for the diagnosis of leptospirosis have low sensitivity during the early stage of the disease. Efforts are being made to develop simpler, effective, efficient, and inexpensive diagnostic methods. The polymerase chain reaction (PCR) has also been used to detect the leptospirosis in farm animals by some researchers (2, 6, 9 11). These studies have proved that PCR is faster and more sensitive than the conventional methods. PCR assay has been used to various clinical specimens such as urine, blood, semen and aborted fetus in order to detect leptospiral DNA. Also PCR may be used for differentiation pathogenic and saprophytic leptospiral (13).

Interstitial Nephritis is rarely recognized as a cause of clinical disease in farm animals although it is a frequent postmortem finding in some species (16). *Leptospora interrogans*, bovine malignant catarrhal fever (MCF), *Theileria parva*, Escherichia coli (E.Coli) and Lumpy skin disease are the causes of interstitial nephritis. The kidney is an important reservoir for *Leptospora interrogans* (16).

Leptospiral infection in Ahvaz, the capital of Khouzestan province, was higher than the other province of Iran. It may be probably due to climate condition, because the weather in Khouzestan is commonly warm and warmer than the other province of Iran. In addition, despite very little annual rainfall, due to many river branches reaching this province, the surface water is higher than it could be expected. It might be expected that more animal become infected in warmer seasons of the year when condition are favorable for survival of leptospires in surface water and animal waste (8). Because of the relatively high prevalence (53.8%) of leptospiral infections in water buffalo in Ahvaz (8), this study was designed to answer the following questions: 1- What is the relationship between antibodies to *L. interrogans* and existing of these bacteria in kidney with shedding from urine? 2- What is the relationship between antibodies to *L. interrogans* and/or existing of these bacteria in kidney with interstitial nephritis? 3- How much interstitial nephritis is related to *L. interrogans* infection?

**Material and Methods**

Totally, 353 slaughtered water buffaloes (143 female and 210 male) were randomly sampled in Ahvaz abattoir in the southwest of Iran, between May to October 2012. Just after slaughtering, blood sample was collected and after opening of carcasses, 10ml of urine sample were directly taken from the bladder by sterile syringe. Then, one piece of both kidneys tissues was picked out. Blood samples were centrifuged and the collected sera were kept at -20°C. Finally, sera were submitted, for MAT, to the leptospira research center of veterinary faculty of Tehran University. Urine samples were transferred to the bacteriological laboratory of veterinary faculty of Shahid Chamran University of Ahvaz and centrifuged at 5000rpm for 10 min. The supernatant was removed so that about 1ml of remaining pellet suspension was stored at -20°C for PCR assay. Kidney samples were immediately put in 10% buffered formalin for thirty days and were embedded in paraffin wax; 5µm thick sections were also stained routinely with Hematoxylin and Eosin (H&E) and two 10µm thick sections were provided into a sterile microtube for PCR assay.

The MAT was performed to detect serum antibodies against *L. interrogans* by use of seven live serovars: hardjo, pomona, grippotyphosa, icterohaemorrhagiae, ballum, tarassovi and australis. Sera were initially screened at a dilution of 1:100 against live antigens. The results were considered positive when 50% or more of agglutination of leptospires were found (14). Positive sera, in 1:100 dilution, were titrated against reacting antigens in serial dilution from 1:100 to 1:800. Individual sera were considered positive that agglutination was found at 1:100 or more dilution.

The method reported by Burhan *et al* (4) was applied for DNA extraction from urine samples. Briefly, 0.6ml of urine sediment was transferred to a sterile microtube and 0.6ml of 1mM, EDTA was added to the pellet suspension. The mixture was vortexed and centrifuged at 13000rpm (Microcentrifuge, Eppendorf, Germany) for 15 min. The supernatant was removed so as to leave 100-200µl in the bottom of tube. The remained pellet suspension was boiled for 15 min and 5µl of its supernatant was used as a template in PCR.

DNA extraction from formalin-fixed and paraffin-embedded kidney tissue samples was carried out using a commercial DNA extraction Kit (DNA extracted kit, Intron, S. Korea), based on manufacturer’s instructions. In brief, two 10µm sections were transferred from each paraffin kidney block into a sterile microtube and deparaffinised in 1.2ml of xylene by vortex for 15 seconds. After centrifuging at 13000rpm for 5 min, the supernatant was discarded and 1ml of absolute ethanol was added to deparaffinised pellet tissue. After removing ethanol, the pellet was dried at room temperature and resuspended in 200µl of kit lysis buffer, mixed by vortexing for 15 seconds and added 20µl of proteinase K solution (20mg/ml). Then, each pellet was incubated at 55°C for 12h. Following, 350µl kit binding buffer was added and completely mixed well by gently vortexing. This mixture was transferred to a spin column filter in a 2ml collection tube and centrifuged at 13000rpm for 1 min. The collecting tube was removed and the column filter was placed back in a new 2 ml collecting tube. Absorbed DNA was washed with 500µl of kit washing buffer A and the
column was centrifuged for one min at 13000rpm. Washing was repeated with washing buffer B. Thereafter, the column filter was placed in a nuclease free microtube, 50μl of elution buffer was directly added onto the filter, and after 1 min incubation at room temperature the tube was centrifuged for 1 min at 13000rpm. Five microliter of extracted DNA was used for the first run PCR as a template.

PCR was implemented by using a gradient thermocycler (Eppendorf, Germany). The external and nested primers (Table I) were LipL32 that encode a prominent lipoprotein in the outer membrane of pathogenic *Leptospira* spp (4).

In the first reaction, 2.5μl of 2X mastermix (Sinagen, Iran), containing each deoxynucleoside triphosphate, MgCl₂ and *Taq* DNA polymerase, 5 μl of template DNA, 1 μl of 10μM of both forward and reverse external primers (Table I) and 5.5μl of nuclease free water in a 25μl volume, were mixed and the reaction was carried out in a PCR thermocycler (Mastercycler, Eppendorf) as follows: a predenaturation at 94˚C for 5 minutes, 35 cycles of 94˚C for 30 seconds, 55˚C for 30 seconds and 72˚C for 30 seconds and a final step of 5 minutes at 72˚C. Beside each set of PCR reaction, a positive control (heat inactivated *L. interrogans* bacterin, supported by Leptospirosis Research Laboratory, University of Tehran) and a negative control (water instead of extracted DNA) were prepared and tested.

Nested reactions were done in a 25μl of the reaction mixture which contained: 12.5 μl of mastermix, 1μl of 10 μM of each internal primer, 9.5μl nuclease-free water, and 1μl of product from the first PCR, under the same thermal conditions as the first reaction.

Eight microliter of second PCR reaction products were evaluated by electrophoresis in a Syber Green (Sinagen, Iran) which contained 1.5% agarose gels in standard conditions. The gels read under UV light and 183 bp-long electrophoresis strips were considered positive.

Statistical analysis was achieved using Chi-Square test which aimed to detect differences or correlation between all variables.

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### Results

The results indicated that 153 out of 353 (43.3%) water buffaloes presented antibodies against one or more examined serovars. Among 153 seropositive buffaloes, 68.6%, 29.4% and 2%, had antibodies against one, two and three examined serovars, respectively. The highest number of reactors was due to hardjo (56.2%), followed by descending pomona (52.3%), australis (9.8%), tarassovi (5.9%), grippotyphosa (4.5%) and icterohaemorrhagiae (3.9%). None of the animals have shown antibody against ballum and the most common mixed infection related to pomona and hardjo was seen in 24 positive sera.

Forty-three out of 353(12.2%) studied buffaloes were positive in PCR, so that, positive PCR products with a molecular size of 183 bp were obtained from 29 (8.2%) of kidney tissues and 17 (4.8%) of urine samples (Fig 1). Three out of 29 buffaloes whose kidney samples were positive in nested PCR were also positive in urine (Table II).

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### Table I: Sequence of primers from the genomic region that encodes LIP L32 of *Leptospira interrogans*

<table>
<thead>
<tr>
<th>Primer sets</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>External</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5’-CGCTTGTGGTGTCTTGGTGGT-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-CTCACCGATTTCGCCTGTTGG-3’</td>
</tr>
<tr>
<td>Internal</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5’-CTCCCAATTTCAGCAGATTACG-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-TTCTGAGCGAGCACAATCCC-3’</td>
</tr>
</tbody>
</table>

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Histopathological results showed that 128 examined animals (36.3%) had renal lesions including the following:
interstitial nephritis (75%), acute tubular necrosis (ATN) (10.1%), hydronephrosis (10.1%), pyelonephritis (3.9%), and glomerulonephritis (0.08%).

Out of 153 (43.3%) buffaloes with positive MAT, 24 cases were positive in nested PCR of kidney and/or urine samples, synchronously. Versus, 19 out of 200 buffaloes, diagnosed as negative by MAT were positive in nested PCR of kidney and/or urine samples. Statistical comparison indicated that there was no significant association between positive results of MAT and nested PCR for diagnosing leptospirosis (P > 0.05) (Table III). According to table III, there is no relationship between MAT, PCR and pathological findings.

Discussion

Various seroepidemiology of bovine and buffalo leptospirosis have been carried out in Iran and other countries. In a previous study in Ahvaz, the prevalence of L. interrogans infection in buffalo was 53.8% and the commonest serovars were canicola and hardjo (8). The prevalence of leptospiiral infection in buffalo from other countries based on serosurvey has been recorded to be 1.7%, 53.9%, 63.6%, and 23% in Nigeria, Brazil, Thailand and Brazil, respectively (1, 12, 15, 19). The most frequent L. interrogans serovars vary somewhat from country to country. For example, L. interrogans serovar tarassovi and hardjo in Nigeria, hardjo and wofli in Brazil, shermani and tarassovi in Thailand were the most common serovars in buffalo (1, 12, 15, 19). Based on these reported results, seroprevalence and commonest serovar of leptospiral infection in buffalo are partly variable in different countries. The causes of these variable prevalence may refer to the difference in sanitary conditions, control efforts as vaccination and environmental factors as humidity, temperature and soil pH, since the L. interrogans survives for a long time in warm and moist environment with an almost natural pH, as the region of the present study.

Various studies carried out to investigate the presence of leptospiral DNA by PCR in the urine and the other samples of farm animals (2, 4, 20). Ten out of 30 urine samples of cattle with leptospirosis, which were formerly positive in urine culture, were positive in PCR, too. But out the other 20 urine samples that were negative in culture, 16 were positive in PCR (2). In the research on prevalence of cattle leptospirosis with PCR of urine and blood samples in Turkey, 9.4% of the urine samples were positive but the blood samples were all negative (20). The PCR surveys which were carried out on urine cattle of Turkey, the prevalence of leptospirosis was reported 4.02% and 9.4% by Burhan et al (4) and Yesilmen et al (20), respectively.

The relationship between MAT and detection of L. interrogans in urine by PCR was investigated in farm animal in Brazil and therefore 45.6% cows, 41.3% equines and 34% goats were positive in MAT, whereas urine PCR of 21.6% cows, 36.2% equines and 77.4% goats was positive (10). The findings of Otaka et al (15) indicated that seroprevalence rate of leptospirosis in cattle was 23%, but 50% of cattle with negative MAT were positive in urine PCR. Sakhaee et al (17) reported that there was not any significant association between MAT and urine PCR.

In the current study, no significant association was observed between interstitial nephritis, MAT and nested PCR. One reason for this result can be related to the serovar type and host-parasite relationships. Because, the pathogenic serovars in maintenance hosts are associated with long-term

<table>
<thead>
<tr>
<th>Urine PCR</th>
<th>Kidney PCR</th>
<th>Urine and Kidney PCR</th>
<th>Urine and/or Kidney PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MAT (+)</td>
<td>12 (3.4%)</td>
<td>14 (4%)</td>
<td>139 (0.5%)</td>
</tr>
<tr>
<td>MAT (-)</td>
<td>5 (1.4%)</td>
<td>15 (4.2%)</td>
<td>185 (0.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>17 (4.8%)</td>
<td>336 (8.2%)</td>
<td>324 (0.8%)</td>
</tr>
</tbody>
</table>

*Microscopic Agglutination Test

Table II: Comparison of obtained results by nested PCR and MAT in studied buffaloes

<table>
<thead>
<tr>
<th>Female</th>
<th>Male</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR+ IN</td>
<td>9</td>
<td>15</td>
<td>24</td>
</tr>
<tr>
<td>MAT+ IN</td>
<td>10</td>
<td>32</td>
<td>42</td>
</tr>
<tr>
<td>MAT+ PCR</td>
<td>4</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>MAT+ PCR+ IN</td>
<td>4</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>(MAT or PCR)+ IN</td>
<td>10</td>
<td>35</td>
<td>45</td>
</tr>
</tbody>
</table>

*Microscopic Agglutination Test

Table III: Comparison of obtained results by nested PCR, MAT, and renal pathology in studied buffaloes
existing in kidney and low antibody response. In contrast, these serovars in accidental hosts lead to a high antibody response and a short kidney phase. On the other hand, the MAT is particularly useful in diagnosis of disease associated with incidental, non-host-adapted serovars or acute disease associated with host-adapted serovars. It is less useful in diagnosis of chronic disease in maintenance hosts since antibody response to infection may be negligible in chronic infections or may persist from subclinical infections (16). Therefore, the animals are negative in MAT and have no antibodies to leptospirosis may be maintenance hosts and bacteria are excreted through the urine and may cause interstitial nephritis. Buffalo may be an accidental host for all the employed serovars. Therefore, leptospiral infection with undergoing serovars in buffalo is expected result in a high antibody response (probably positive MAT) and a short kidney phase (probably negative PCR).

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Conflict of interest

The authors have no conflict of interest

References


